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Full Length Research Paper

Endophytic and rhizospheric bacteria from *Opuntia ficus-indica* mill and their ability to promote plant growth in cowpea, *Vigna unguiculata* (L.) Walp

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Studies on the biodiversity of plants associated microorganisms play an important role in food production. Ficus-tree, *Opuntia ficus-indica* Mill, are cultivated in many nations worldwide intercropping with other cultures, like cowpea, *Vigna unguiculata* (L.) Walp. The objectives of this study were to isolate and determine the diversity of bacterial endophytes and rhizobacteria in cacti and their ability to solubilize phosphate, the *nif* presence, their ability to produce indole-acetic acid (IAA) and verify their efficacy to promote plant growth in cowpea plants. Bacteria were isolated from rhizospheric soils, from surface- sterilized stems and roots of plants collected in Brazil, in nitrogen- free medium, and identified through fatty acid analysis. Gram-negative bacteria comprised 63% of endophytic bacteria and 80% of rhizobacteria. Only 13% showed amplification of *nifH* gene. From the 68 bacterial strains, associated with cacti, 18% produced IAA, and the best results were observed in *Agrobacterium radiobacter, Klebsiella trevisanii, Enterobacter agglomerans* and *Paracoccus denitrificans.* Phosphate solubilization was observed in 6% of strains. None of the strains inoculated in cowpea plants promoted stem and foliar growth. Four percent of strains showed ability to increase the root dry matter compared to the control group (inoculated with a pool of indigenous semi-arid *Rhizobium*).

Key words: Endophytes, rhizosphere, indole-acetic acid (IAA), phosphate solubilization, cacti, *Vigna*.

INTRODUCTION

Microbial diversity assessments presume that the number of interacting species can be identified, classified and their respective activities extrapolated to their function in different agricultural systems. New species and genera have been continuously reported from a wide geographic range. Endophytic bacteria and fungi have probably developed intimate relationships with their host plants through co-evolutionary processes and may influence the plant physiology in ways not yet fully elucidated (Mano et al., 2008).

Endophytic bacteria have been found in several parts of the studied plants, and most of these organisms appear to originate from the rhizosphere or phyllosphere; however, some may be transmitted through the seed. Endophytic bacteria could promote plant growth and yield by enhancing soil fertility through phosphate solubilization and nitrogen fixation (Ryan et al., 2007).

It is well known that a considerable number of bacterial species, mostly associated with the plant rhizosphere, are able to exert a beneficial effect on plant growth. This group of bacteria has been termed plant growth promoting rhizobacteria (PGPR). The mechanisms through which PGPR may stimulate plant growth are probably due to the production of indole-3-acetic acid (IAA) and cytokinin-like compounds as well as by lowering ethylene levels in plants (Ali et al., 2009); fixing nitrogen (Zaidi and Mohammad, 2006), and increasing

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the availability of nutrients such as phosphate (Malboobi et al., 2009).

Free bacteria in soil and rhizosphere, including phytopathogenic, epiphytic, and PGPRs, produce IAA (Ali et al. 2009). The indole-acetic acid (IAA) biosynthesis may occur through different metabolic routes and leads to accumulation of this hormone in the plant tissues and to an increased biomass (Arkhipova et al., 2007). Bacteria able to produce and release IAA are potential plant growth promoters (Ali et al., 2010), increasing the root surface and making a larger infection area for potential diazotrophic bacteria colonization (Molla et al*.,* 2001). It has long been claimed that the microbial synthesis of plant growth regulators is an important factor in soil fertility.

PGPR promotes changes in auxin balance in host plant, and this influences nodule organogenesis (Spaepen et al., 2009) and genes expression in the *Rhizobium*-legume interaction that induces symbiotic associations between leguminous plants and rhizobia with positive effect on the growth and yield of legumes (Shaharoona et al., 2006).

The biological fixation of N_2 is catalyzed by the nitrogenase complex, which transforms N_2 into NH_4^+. This latter is composed of two metalloproteins present in most part of the N_2-fixing bacteria. The involved metalloproteins are the dinitrogenase, a MoFe protein and the reductase dinitrogenase, a Fe protein, respectively codified by *nif*D and *nif*H genes (Howard and Rees, 1996).

The analysis of the profile of cell wall fatty acids has been widely employed in studies on rhizospheric (Çakmakç et al., 2010; Misko and Germida, 2002) and endophytic microbial communities (Dias et al., 2009). This analysis was based in fact that some bacterial groups present unique lipidic profiles (Sasser, 1990).

Desert plants, especially cactus, could be used to prevent soil erosion and also contribute for the revegetation of disturbed desert areas, but, these plants present low establishment rates and slow growth after being transferred from natural habitats. Puente et al. (2004a) shows that native arid zone rhizoplane bacteria, in special *Bacillus* species, presented plant growth promoting effect in cactus growing under growth chamber conditions, and may be applied for cultivating cacti in arid ambient for revegation programmes of eroded areas. About 12% of Brazil's surface, especially the Northeast, is subject to severe rainfall deficiency and temperatures are relatively high which can reach up to 50ºC. Under these conditions, Cowpea [*Vigna unguiculata* (L.) Walp.] represents 23% of national beans production, and the Northeast Region is responsible for 50% of production and 60% of the planted area (Ferreira et al., 2002). These areas were located where drought is frequent and cultivation occurs as a monoculture or in association with xerophytic plants, for example, the consortium with the prickly-pear, *Opuntia ficus-indica* Mill (Albuquerque and Rao, 1997).

The current investigation was therefore aimed to select bacteria from the semi-arid region, associated with *Opuntia ficus-indica* Mill, which promote plant growth of cowpea (*Vigna unguiculata*) or increase the symbiotic potential of local rhizobial strains.

MATERIALS AND METHODS

Places of collection

Plants were collected in two regions of Brazil; one in the semi-arid Northeastern region and another in the state of São Paulo (Queimadas – PB, S 07°19' 42,3'' and W 35°53' 48,1 ''; Taperoá – PB,S 07°06' 58'' and W 36°44' 56,1''; Patos – PB, S 07°02' 33,5'' and W 37°21' 34,7''; São José da Tapera – AL, S 09°32' 51,9'' and W 03°72' 13,3''; Valinhos – SP, S 22°43' 36,3 '' and W 46°58' 56''). In Northeastern region, two states were selected for their low precipitation: Paraíba (PB) and Alagoas (AL).

Isolation of endophytic bacteria

Roots and stems (nopals) of healthy plants were surface sterilized with 70% ethanol for 1 min and 2.5% sodium hypochlorite to disinfect surfaces for 20 min, previously standardized; and then in 70% ethanol for 30 s, following three successive washes in distilled sterilized water. All extremities of the materials were discarted, and the nopals were broken up in cubes of 0.5 cm. of arest. The fragments were transferred into solid NFb medium (Dobereiner et al*.*, 1995): malic acid, 5.0 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2.2H_2O$, 0.02 g; micronutrients solution, 2 ml; bromotimol blue (0.5% in 0.2N KOH), 2 ml; FeEDTA (1.64%), 4 ml; vitamin solution, 1 ml; KOH, 4.5 g. pH was adjusted to 6.5 or 6.8 with NaOH, the volume is completed to 1000 ml with distilled water and 15 g of agar was added. Micronutrient solution: $CuSO_4.5H_2O$, 0.04 g; $ZnSO_4.7H_2O$, 1.2 g; H_3BO_3, 1.4 g; $Na_2MoO_4.2H_2O$, 1 g; $MnSO_4.H_2O$, 1.175 g. Complete volume to 1 L with distilled water. Vitamin solution: biotin, 10 mg; piridoxol-HCl, 20 mg. Dissolve in water bath, complete the volume to 100 ml with distilled water and keep solution in refrigerator. The material was incubated at 28°C, with daily observation.

Isolation of rhizospheric bacteria

From each site, three healthy plants were harvested, the roots were excised, and the adhering soil was separated from the roots. The soil samples (1 g each) were vigorously shaken in 9 ml of autoclavated saline solution (NaCl 0.85% in water) for 2 min by using a Vortex mixer. Serial dilutions of suspensions were plated onto solid NFb medium (Dobereiner et al*.,* 1995), which is known to be selective for the recovery of nitrogen fixers. The plates were incubated at 28°C. Individual colonies were picked and transferred to new NFb medium plates.

Strain identification

Each strain was identified through the analysis of fatty acid methyl-esters (FAMEs) using the Microbial Identification System developed by Microbial ID (MIDI, Newark, DE). Cellular fatty acids were extracted according to the method of Sasser (1990). Fatty acid methyl-esters from each strain were separated using a Hewllett-Packard gas chromatograph model adjusted with a fused silica capillary column (25 m x 0.2 mm internal diameter). FAME peaks were named by the MIS software, and the bacterial strains were

Table 1. Characteristics of soil used for the plant growth promotion experiment.

Variable	pH in water	P	K	Ca	Mg	Al	O.M.	Sand	Silt	Clay
		(mg/dm^3)"		(cmoVdm^3)			(dag/kg)	%		
Soil	6.5	6.6	33.3	1.3	0.66	0.1	1.24	66	13	19

identified using the MIS "Aerobia Library" (Version TSBA50).

Screening of bacteria for IAA production

Endophytic bacteria and rhizobacteria isolated from roots and rhizosphere of cacti were evaluated for auxin (indole-3-acetic acid) production. Sixty-eight bacterial strains were grown on solid NFb medium (Döbereiner et al*.,* 1995) supplemented with 10mM NH_4Cl and 10 mM L-tryptophan. Each bacterial strain was inoculated in equidistant point on nitrocellulose membranes placed on the agar surface. The cultures were incubated for 48 h in the dark. After this period, the membranes were removed and immerged into Salkowsky reagent (Bric et al., 1991). *Gluconacetobacter diazothophicus* BR11281 was used as positive control. Producer strains react with Salkowsky reagent to yield a pink-colored product after 30 min of incubation.

IAA determination

The IAA production was measured in NFb liquid medium, supplemented with 10 mM NH_4Cl and 10 mM L-tryptophan. The cultures were incubated in the dark at 28℃, under sh aker (120 rpm), for 26 h. Bacterial cells were removed from the culture by centrifugation at 5000 g for 10 min. The IAA present in the supernatant was assayed according to the standard method modifief from Rahman et al. (2010) in which the hormone present in the culture reacts with Salkowski reagent, which was quantitatively measured on a spectrophotometer at 530 nm. Kinetics of the IAA production was calculated from a standard curve and expressed as IAA equivalents by ml (IAA µg/ml). Comparative experiments were performed twice, each one with four replicates per treatment.

***nif* genes detection**

The selection of bacteria that code for genes nifH (19F 5' GCIWTYTAYGGIAARGGIGG-3'; 407R 5'-AAICCRCCRCAIACIACRTC-3') (Ueda et al., 1995) and nifD (FdB261 5'-TGGGGICCIRTIAARGAYATG-3'; FdB260 5'-TCRTTIGCIATRTGRTGNCC-3' (Stoltzfus et al., 1997) was made by amplifying it by PCR using specific primers. The extraction of total genomic DNA were performed according Sambrook et al. (1989). PCR reactions were performed in a final volume of 25 ml. Containing 0.5 - 10 ng of template DNA, 1 M of each primer, 0.2 mM of each dNTPs, 1.5 mM MgCl2, 2, 5 ml of enzyme buffer (10X), 0.5 U / ml of Taq DNA polymerase (Life Technologies). In all reactions were used seven positive controls, with the template DNA of bacterial strains provided by Embrapa Agrobiology Center (*Azoarcus indigens* VB 32 T, *Azospirillum amazonense* BR111 42, *A. irakense* AGROB, *A. lipoferum* BR11080, *Burkholderia vietnamiensis* TVV 75, *Gluconacetobacter diazotrophicus* BR11281, e *Herbaspirillum rubrisubalbicans* BR11192), and a negative control without DNA template. The amplification reaction was carried out in Peltier PTC200 thermal cycler programmed to perform an initial denaturation of 5 min at 94℃, 40 cycles of 30 s to 94℃, 1 min at 59℃, 30 s and more 72℃ and a final extension of 5 min at 72℃. Ten microliters of PCR reaction we re observed in agarose gel 1.2%, together with the molecular weight marker 100 bp DNA Ladder (Life Technologies).

***In vitro* phosphate solubilization**

The selection of bacterial strains isolated from *Opuntia ficus-indica*, able to solubilize phosphate in vitro was performed by cultivating in plates containing media from the following methods: Pikovskaya agar (PVK) (Pikovskaya, 1948), NBBIY agar (Nautiyal, 1999) and a NBRIP agar (Nautiyal, 1999). After 14 days of incubation at 28℃, the diameter of the colony and the diameter of the halo of phosphate solubilization were measured. The size of the halo of solubilization was obtained by subtracting the value of the colony diameter from the total halo solubilization diameter (Nautiyal, 1999).

Plant growth promotion assay

The plant growth promotion of cowpea and stimulation of nodulation was tested only for strains that showed amplification for the segment of at least one *nif* gene. The growth of these strains was carried out in nutrient broth, at 28ºC on shaker at 120 rpm for 24 h. After this period, bacterial cells were centrifuged at 1500 rpm, and washed in saline (0.85% NaCl in water). Cowpea seeds of a landrace maintained by Paraiba farmers were superficially disinfected with sodium hypochlorite 25% for 5 min and 70% ethanol for 1 min. These seeds were inoculated with a bacterial suspension (10^8 CFU) for 1 h and then sown in pots (5L) containing non-sterile substrate composed of equal parts of sand and soil (dystrophic latosoil), from an area pasture with Braque. Soil was collected at a depth of 20 to 40 cm, after removing the upper layers. This was analyzed chemically and physically according to the methodology proposed by EMBRAPA (1997) whose results are shown in Table 1, added with soil from cowpea areas at the semiarid Paraiba, acting as a pool of rhizobia. The experiment was entirely randomized in three replicates, each pot contained 20 seeds. After reviewing emergency were left 10 plants per pot. After 60 days the plants were removed carefully, washed and stored under refrigeration. The variables studied were: the emergence of seeds, monitored daily, the weight of the dried roots and shoot dry weight, number of nodules and dry weight of nodules.

Statistical analysis

Statistical analysis was performed by SPSS (Statistical Package for Social Sciences) 11.0 for windows. The variables were submitted to descriptive analysis expressed by the mean and standard deviation, after the descriptive analysis was performed to T-test comparison of means.

RESULTS

Bacterial diversity

A total of 37 endophytic bacteria from the plant segments

and 31 rhizobacteria were isolated from cacti and rhizospheric soil, respectively, in free nitrogen medium. The endophytic bacteria and the isolated PGPRs comprised each one 12 genera. Bacteria not identified through the MIS software represented 29% of the total. The commonly isolated genera from roots and stems were *Enterobacter* and *Bacillus;* and from rhizosphere were *Ochrobactrum.*

The differentiation of specific genera indicated that in the rhizospheric populations of *Ochrobactrum* and *Acinetobacter* were the most prominent group with 18.2%, followed by *Stenotrophomonas* with 13.6% (Table 2).

The taxonomic identification of the 49 strains revealed a predominance of Gram-negative species (71.42%). Among them, members of the genera *Pantoea, Enterobacter, Acinetobacter* and *Stenotrophomonas* predominated. Gram-positive strains occurred in both roots and rhizosphere, specially, the genus *Bacillus.*

Bacterial IAA biosynthesis ability

High IAA biosynthesis with L-tryptophan as precursor occurred in nine bacterial strains, with IAA production ranging from 3.5 to 17.45 $\mu g.ml^{-1}$ (Table 3). The strains considered as a hyper producer *Gluconoacetobacter diazotrophicus* bacterium produced, in these conditions, 4.32 $\mu g.ml^{-1}$ of IAA.

Other bacterial genera also evaluated produced high amount of the hormone, including *P. denitrificans, E. agglomerans* and *Painibacillus pabuli. O. antropi, Rhodococcus erytropolis* and *B. lentimorbus* produced detectable amounts of IAA. It is verified that a great number of endophytic bacteria produced higher amounts of auxins.

***nif*H gene detection**

Only nine strains amplified the *nif*H (Table 4) and no amplified *nif*D gene. Probably these strains are not able to make the biological fixation of N_2. All strains used as positive control genes were amplified *nif*H and *nif*D. The taxonomic identification of lineages that encode the *nif*H, made by analysis of fatty acid profile, grouped these bacteria in the genera *Ochrobactrum*, *Stenotrophomonas*, *Rhodococcus*, *Citrobacter*, *Pseudomonas* and *Enterobacter* (Table 4).

Phosphate solubilization

Phosphate solubilization was observed in strains positive for the presence of *nif*H, *E. agglomerans* (NFCA1F) solubilized in three media tested, *Ochrobactrum anthropi* (RINFA1B) and *Stenotrophomonas maltophilia* (RINFA2K) solubilized phosphate only in PVK medium, and *Pseudomonas* sp. (NFCA1C) only in NBRIY medium (Table 4).

***Vigna unguiculata* plant-growth promotion**

All bacterial inoculants tested stimulated secondary roots nodulation, except the *Citrobacter freundii*. This increase in the expression of nodulation arrived, in some cases, to 576%. It was found that the species *Ochrobactrum anthopi* (RINFA2A), *R. erytropolis* (RINFA2I), *S. maltophilia* and *O. anthopi* (RINFA2N) increased by 31.4, 30.2, 18.3 and 37.5%, respectively, the total number of nodules. The two strains of *O. anthopi* quoted above also favored the development of the plant, as well evidenced by plant height and dry weight of the same (Table 5). The weight of roots was also increased as a result of inoculation of bacteria to seeds, highlighting the most significant species *O. anthopi* and *R. erythropolis*, and these strains producing IAA.

DISCUSSION

Cactus roots colonization by microorganisms are dependent of the root age, in young roots present a heavily colonization, while the majority of older roots lacked detectable microbe population (Puente et al. 2004b). What was also observed in our study that made use of roots with more than a year, chosen due the absence of new roots.

Some endophytic and rhizospheric bacteria isolated from cacti bear many similarities to bacteria found in other host plants. In many previous studies in which the taxonomic status of the endophytes was determinated, members of the genus *Pseudomonas* have predominated. In this study, no strain of this genus was isolated. Perhaps, the environmental conditions observed in semi-arid regions of Northeastern region of Brazil limit the abundance of *Pseudomonas.* On the other hand, it is very common the adaptation of other genera, such as *Bacillus* and *Nocardiaceae,* tolerant to hydric stresses.

Bacterial communities from internal tissues of cacti were distinct from those of rhizosphere. The Proteobacteria presented the largest fraction of strains (63.26%) with members of α and y subdivisions. Proteobacteria were a predominant component of the community associated with cacti roots. Components of the β-Proteobacteria, were not found.

In this study we corroborated with others data that demonstrate as *Bacillus* spp. are frequently isolated from the rhizosphere of plants, and also common endophytes (Calvo-Velez et al., 2010; Mehta et al., 2010; Araujo et al., 2001). Due to their spore forming capability they are readily adaptable to environmental conditions and are common in the semi-arid regions associated with the

Table 2. Bacterial strains associated with *Opuntia ficus-indica*, isolated in free nitrogen medium.

Class	Species		Endophytes		Rhizosphere	Collection point
			Roots	Nopals		
α - Proteobacteria	*Agrobacterium radiobacter*	NCOL12	+	-	-	Valinhos
	Agrobacterium radiobacter	NCOL15	+	-	-	Valinhos
	Brevundimonas vesicularis	RINFA2G	-	-	+	S. J. do Tapera
	Ochrobactrum antropi	RINFA1A	-	-	+	S. J. do Tapera
	Ochrobactrum antropi	RINFA1B	-	-	+	S. J. do Tapera
	Ochrobactrum antropi	RINFA2A	-	-	+	S. J. do Tapera
	Ochrobactrum antropi	RINFA2N	-	-	+	S. J. do Tapera
	Paracoccus denitrificans	NCOL13	+	-	-	Valinhos
	Paracoccus denitrificans	NCOL123	+	-	-	Valinhos
	Sphingomonas paucimobilis	NFCA1C	-	+	-	S. J. do Tapera
γ - Proteobacteria	*Acinetobacter calcoaceticus*	RINFA1C''	-	-	+	S. J. do Tapera
	Acinetobacter calcoaceticus	RINFA1E	-	-	+	S. J. do Tapera
	Acinetobacter johnsonii	RINFA1E'	-	-	+	S. J. do Tapera
	Acinetobacter johnsonii	RINFA1E''	-	-	+	S. J. do Tapera
	Citrobacter freundii	NCOL27'B	+	-	-	Queimadas
	Enterobacter agglomerans	NFCA2'	-	+	-	S. J. da Tapera
	Enterobacter agglomerans	NFRT3A	+	-	-	Taperoá
	Enterobacter agglomerans	NFCA1F	-	+	-	S. J. da Tapera
	Enterobacter agglomerans	NCOL2RI1	-	-	+	Queimadas
	Enterobacter agglomerans	NCOL117	+	-	-	Valinhos
	Escherichia coli	NFRT3E	+	-	-	Taperoá
	Flavimonas oryzihabitans	RINFA1J	-	-	+	S. J. da Tapera
	Klebsiella trevisanii	NFRT3C	+	-	-	Taperoá
	Kluyvera criocrescens	NCOL211	+	-	-	Queimadas
	Pantoea ananás	NCOL2RI3	-	-	+	Queimadas
	Salmonella typhimurium	NCOL27B	+	-	-	Queimadas
	Stenotrophomonas maltophilia	RINFA1A'	-	-	+	S. J. da Tapera
	Stenotrophomonas maltophilia	RINFA1B'	-	-	+	S. J. da Tapera
	Stenotrophomonas maltophilia	RINFA2K	-	-	+	S. J. da Tapera
	Stenotrophomonas maltophilia	NCOL11	+	-	-	Valinhos
	Stenotrophomonas maltophilia	NCOL113	+	-	-	Valinhos
Eubactéria	*Bacillus coagulans*	NCOL12RI7	+	-	-	Valinhos
	Bacillus lentimorbus	NCOL29	+	-	-	Queimadas
	Bacillus megaterium	NFRP3B	+	-	-	Patos

Table 2. Contd.

	Bacillus megaterium	NFRP3C	+	-	-	Patos
	Bacillus megaterium	NFRP3D	+	-	-	Patos
	Bacillus megaterium	NCOL28	+	-	-	Queimadas
	Bacillus megaterium	NFCT3D	-	+	-	Taperoá
	Bacillus megaterium	NCOL18	+	-	-	Valinhos
	Chryseobacterium balustinum	RINFA2F''	-	-	+	S. J. da Tapera
	Chryseobacterium indologenes	RINFA1C'	-	-	+	S. J. da Tapera
	Chryseobacterium indologenes	NCOL14	+	-	-	Valinhos
	Paenibacillus macerans	NCOL28'	+	-	-	Queimadas
	Paenibacillus pabuli	NCOL210	+	-	-	Queimadas
	Paenibacillus polymyxa	RINFA2D	-	-	+	S. J. da Tapera
Actinobacteria	*Nocardia otitisdiscariarum*	RINFA1'	-	-	+	S. J. da Tapera
	Rhodococcus erytropolis	RINFA2I	-	-	+	S. J. da Tapera
	Rhodococcus globerulus	RINFA2J	-	-	+	S. J. da Tapera

Table 3. Quantification of the IAA production by bacterial strains associated with *Opuntia ficus-indica* Mill.

Strain	Explant	IAA production(µg/ml)
Ochrobactrum antropi (RINFA1A)	Rhizosphere	0.0765 ±0.003[c]
Rhodococcus erytropolis (RINFA2Ì)	Rhizosphere	0.159 ±0.008[c]
Ochrobactrum anthropi (RINFA1B)	Rhizosphere	0.8065 ±0.026[c]
Bacillus lentimorbus (NCOL29)	Root	0.89 ±0.017[c]
Acinetobacter calcoaceticus (RINFA1C '')	Rhizosphere	3.5275 ±0.046[b]
Paenibacillus pabuli (NCOL210)	Root	4.1 ±0.015[b]
Gluconacetobacter diazotrophicus (standard)*		4.328 ±0. 06[b]
Enterobacter agglomerans GC sub-group IV (NCOL117)	Root	5.945 ±0.018 [b]
Paracoccus denitrificans (NCOL13)	Root	6.485 ±0.005[b]
Not identified bacteria (RINFA2L)	Rhizosphere	6.76 ±0.004[b]
Enterobacter agglomerans GC sub-group III(NCOL2RI1)	Rhizosphere	8.6745 ±0.021[b]
Klebsiella trevisanii (NFRT3C)	Root	10.75 ±0.051[a,b]
Agrobacterium radiobacter (NCOL12)	Root	15.45 ±0.039[a]
Escherichia coli GC C sub-group (NFRT3É)	Root	17.45 ±0.034[a]

*Strain of *G. diazotrophicus* kindly donated from Embrapa Agrobiologia, Rio de Janeiro, Brazil. Observation – Values followed by the same letter are statistically equal.

Opuntia cholla (Puente et al., 2004b). When isolation was performed in NFb medium, *Bacillus* strain were only obtained as endophytes. Members of the genus *Bacillus* are described as potential plant growth promoters producing indole acetic acid (Swain et al., 2007) and values over 17 µg ml^{-1} , in luria bertani broth supplemented with 100 µg ml^{-1} of tryptophan, siderophore, HCN, and solubilizing P, in Pikovskaya broth (Wani et al., 2007).

The amount of auxins found in culture filtrates of *Pseudomonas* and *Acinetobacter* strains, isolated as phosphate-solubilizing bacteria from the rhizosphere of wheat and rye, varies from 0.01 to 3.98 mg indole-3-acetic acid (IAA) equivalent per ml of culture medium and

Table 4. Identification of bacteria isolated from *Opuntia ficus-indica* that encode the *nif*H gene, production of IAA and solubilization.

Strains	Origin	IAAdetection	Phosphate solubilization
Rhizospheric			
Ochrobactrum anthropi (RINFA1A)	São José da Tapera, AL	+	-
Ochrobactrum anthropi (RINFA1B)	São José da Tapera, AL	+	+
Ochrobactrum anthropi (RINFA2A)	São José da Tapera, AL	-	-
Ochrobactrum anthropi (RINFA2N)	São José da Tapera, AL	-	-
Stenotrophomonas maltophilia (RINFA2K)	São José da Tapera- AL	-	+

Table 5. Effect of inoculation of cowpea (*Vigna unguiculata*) with bacteria associated with *Opuntia ficus-indica* Mill, isolated in NFb media and carrying the *nif*H gene on nodulation, biomass of shoots and roots.

Tratment	Axial root Nodules numbers	Secondary roots nodules numbers	Total nodules number per plant	Nodules dry matter mg.pl^{-1}	Plant height cm	Roots dry matter mg.pl^{-1}	Aerial parts dry matter mg.pl^{-1}
Control	13.87± 4.83	0.133± 0.73	14.00± 4.71	128.8± 18.99	14.33± 1.68	1263.33±165.02	5923.3± 425.9
Citrobacter freundii (NCOL27´B)	14.7± 5.05	0.667± 1.81	15.36± 4.85	87.7± 17.69	12.37*± 1.27	1240.0±356.8	5403± 207.9
Pseudomonas sp. (NFCA1C)	9.8*±4.47	5.07*± 4.8	14.9± 5.62	172.8± 54.77	10.7*± 1.46	1456.7±115.9	5730.0± 405.8
Enterobacter agglomerans (NFCA1F)	15.47±5.22	0.9*± 1.30	16.37± 5.48	110.8± 15.55	10.52*± 1.08	1360.0± 55.68	5220.0± 226.5
Ochrobactrum anthropi (RINFA1B)	11.5*± 4.03	2.17*± 3.64	13.67± 5.1	107.4± 16.95	9.53*± 0.82	4186.7*± 503.32	5163.3± 583.8
Ochrobactrum anthropi (RINFA2A)	15.07± 5.66	3.33*± 3.65	18.4*± 6.66	141.2± 17.77	14.667± 1.73	3530.0*± 476.9	5290.0±1974.7
Rhodococcus erytropolis (RINFA2I)	16.27± 4.81	1.97*± 2.97	18.23*± 4.75	144.0± 44.98	10.5*± 1.10	2496.7*± 183.4	5406.7± 907.9
Stenotrophomonas maltophilia (RINFA2K)	10.97± 1.47	0.967*± 1.71	16.57*± 4.61	102.1± 12.2	10.92*± 1.47	1456.67± 116.76	5610.0±153.9
Ochrobactrum anthropi (RINFA2N)	16.4*± 4.57	2.87*± 3.86	19.27*± 6.45	202.7± 45.7	12.08*± 1.18	1716.7± 351.04	5743.3±176.1
Ochrobactrum anthropi (RINFA1A)	12.77± 4.77	1.6*± 1.96	14.37± 4.88	116.6± 29.14	13.72± 1.49	1253.33± 198.58	6053.3± 132.0

* Treatments that differ significantly at 5%, compared to control.

Bacillus thuringiensis in entrapped in k-carrageenan was capable of producing IAA in the presence of rock phosphate ore (RPO) with an average of 6.9 mg/ml of IAA. This value increased when media was supplemented with tryptophan to 13.9 and 20.7 mg/L per batch on RPO-free media (Vassilev et al., 2007). *Rhodococcus*, especially virulent strains, are able to produce IAA (Vandeputte et al., 2005).

It is interesting to note that except for the species *C. freundii*, all bacteria with the potential to fix N_2 were isolated from the Alagoas state near the São Francisco river, with predominantly high temperatures and low rainfall. This is an area extremely impoverished and devoid of technical assistance to farmers, enabling the maintenance of basic techniques without the input of N_2 in the form of fertilizers (organic or chemical), which may have favored the selection and maintenance of bacterial strain.

The predominant species n the rhizosphere of

prickly-pear and carries the *nif*H was *Ochrobactrum anthropii*, an alpha-Proteobacteria group Rhizobialle, where are located some nitrogen-fixing of commercial use. *Ochrobactrum* sp has been reported as a rice endophyte (Verma et al., 2004).

It has been verified in literature (Newton, 2005; Xie et al., 2006) that the species *C. freundii* carrying the *nif*H, also serves on biological nitrogen fixation.

The biological nitrogen fixation has been described for the genus *Enterobacter* and others N_2-fixing bacteria isolated from the sugarcane rhizosphere (Boddey et al., 2003). Since the strain of *Rhodococcus* that encodes the *nif*H observed in this study, was also studied by Rösch et al. (2002).

Instead the characteristic of PVK, a medium developed for the phosphate solubilization by enterobacteria (Pikovskaya, 1948; Nautiyal, 1999) we obtained good results with Bacilli strains. Other studies observed phosphate solubilization by *Enterobacter* and *Pseudomonas* (Pandey et al., 2002; Nautiyal, 1999).

Vigna unguiculata inoculation with bacterial strains originally associated with *Opuntia ficus-indica* carrying the *nif*H gene showed behaviors already described in literature which has significantly increased nodulation in secondary roots, due to the increase of lateral roots and root hairs, penetration sites for *Rhizobium* spp and *Bradyrhizobium* spp, natural nodulating bacteria. Thus, the strains of *S. maltophilia* (NCOL113), *O. anthropi* (RINFA1B), *O. anthropi* (RINFA2A) and *Rhodococcus erythropolis* (RINFA2I) increased dry weight of roots and number of nodules on secondary roots. Similar results were obtained in assays developed under greenhouse conditions relate improved plant yield and plant health with respect to increase in root wet weight and nodulation when co-inoculated with nodule endophytes, compared to inoculation with rhizobia alone (Bai et al., 2002, 2003). Bacterial co-inoculants that stimulate rhizobial colonization was currently claimed "helpers" and promote growth and nodulation of the host plant, we may cite the example of the genus *Bacillus* (Verma et al., 2010).

O. anthropi (RINFA1B) and *O. anthropi* strains (RINFA2A) showed the best results in the nodulation of secondary roots that may have occurred because species of the genus *Ochrobactrum* have the nifH and nodD gene sequences very similar to *Rhizobium* besides being able to effectively nodulate *Lupinus albus* (Trujillo et al., 2005). Root nodule bacterial strains isolated from nodules of *Acacia mangium* grown in the Philippines and Thailand include members of the genus *Ochrobactrum*. One of them induced root nodules on *A. mangium*, *A. albida* and *Paraserianthes falcataria*. These nodules fixed nitrogen and the morphology is their same as those of nodules formed by the isolates (Ngom et al., 2004).

The increase in root weight, in particular, the total nodule appears to be related to the significant reduction in dry weight of shoot, which agrees with Voisin et al. (2007) postulates that the shift in the use of photosynthates to the production and functionality of nodules in detriment of the area leaf and air portion. The best results for the increased nodulation in secondary roots, as well as weight gain root were observed when inoculated rhizosphere isolates. No correlations were found between the biosynthesis of IAA, and phosphate solubilization with these results.

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